Interactions Among Genes Regulating Ovule Development in *Arabidopsis thaliana*

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ABSTRACT

The *INNER NO OUTER* (*INO*) and *AINTEGUMENTA* (*ANT*) genes are essential for ovule integument development in *Arabidopsis thaliana*. Ovules of *ino* mutants initiate two integument primordia, but the outer integument primordium forms on the opposite side of the ovule from the normal location and undergoes no further development. The inner integument appears to develop normally, resulting in erect, unitegmic ovules that resemble those of gymnosperms. *ino* plants are partially fertile and produce seeds with altered surface topography, demonstrating a lineage dependence in development of the testa. *ant* mutations affect initiation of both integuments. The strongest of five new *ant* alleles we have isolated produces ovules that lack integuments and fail to complete megasporogenesis. *ant* mutations also affect flower development, resulting in narrow petals and the absence of one or both lateral stamens. Characterization of double mutants between *ant, ino* and other mutations affecting ovule development has enabled the construction of a model for genetic control of ovule development. This model proposes parallel independent regulatory pathways for a number of aspects of this process, a dependence on the presence of an inner integument for development of the embryo sac, and the existence of additional genes regulating ovule development.

In recent years, the approach of isolating large numbers of mutants affecting floral development followed by examination of genetic interactions among these mutations has led to the formulation of a highly predictive model of determination of floral organ identity in *Arabidopsis thaliana* and *Antirrhinum majus* (reviewed in COEN and MEYEROWITZ 1991; MA 1994; WEIGEL and MEYEROWITZ 1994; YANOFSKY 1995). Much less is known, however, about a critical late aspect of this process: the genetic control of ovule development.

As the site of megasporogenesis, megagametogenesis and fertilization, and as the progenitors of seeds, ovules play a series of critical roles in plant sexual reproduction. In angiosperms, ovules are components of the gynoecium, the set of organs occupying the center of the flower. The gynoecium is composed of unit structures, the carpels, which may form free pistils or fuse together into a single compound pistil. The pistils consist of a basal ovary and a style, which commonly terminates in a glandular stigma. Although ovules develop within the ovaries, the fact that they evolved hundreds of millions of years before the evolution of carpels and ovaries (STEWART 1983) indicates that they should be considered separate organs. While small in size, ovules of angiosperms comprise several morphologic parts including a nucellus (megasporangium), one or two integuments (which subsequently develop into the seed coat), and a funiculus (which connects the ovule to the ovary wall) (ESAU 1965; GASSER and ROBINSON-BEERS 1993; REISER and FISCHER 1993).

The recent identification of mutants affecting ovule development in Arabidopsis, together with construction of transgenic petunia lines with altered ovules (ANGENENT *et al.* 1995; COLOMBO *et al.* 1995), has begun to shed light on the genetic regulation of this process. The majority of mutations isolated to date produce defects in integument development.

Mutations at the recently described *aintegumenta* (*ant*) locus lead to the near complete absence of integuments (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996). Early in ovule development, *ANT* is expressed specifically in the chalaza, the region from which the integuments will emerge, consistent with an early role in promoting integument formation (ELLIOTT *et al.* 1996). *ant* mutations also result in pleiotropic effects on petal shape and stamen number (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996). Consistent with these additional effects, *ANT* was also found to be expressed in floral organ primordia (ELLIOTT *et al.* 1996). The *ANT* gene was shown to encode a protein with similarity to the product of the *APETALA2* (*AP2*) gene (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996). *AP2* is known to have a role in petal identity and organ number. This, together with the observation of synergistic effects of the two genes in *ant ap2* double mutants, indicates that there is some overlap in the functions of these two genes (ELLIOTT *et al.* 1996).

aberrant testa shape (*ats*) and *bell 1* (*bel1*) mutants pro-

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duce ovules with single structures in place of the two integuments (ROBINSON-BEERS *et al.* 1992; LEON-KLOOSTERZIEL *et al.* 1994; MODRUSAN *et al.* 1994; RAY *et al.* 1994). In *ats* mutants, layers of both integuments are found in the single structure that forms, indicating that this structure may represent a fusion of the two integuments (LEON-KLOOSTERZIEL *et al.* 1994). In contrast, integument identity in *bel1* mutants appears to be completely lost, and the single structure formed develops into an aberrant collar of tissue, which sometimes develops further into a complete ectopic carpel (ROBINSON-BEERS *et al.* 1992; MODRUSAN *et al.* 1994; RAY *et al.* 1994). *BEL1* encodes a putative transcription factor containing a homeodomain, and the timing and pattern of *BEL1* expression correlate with establishment of the integument primordia (REISER *et al.* 1995).

superman (*sup*, also known as *floral mutant 10* or *flo10*) and *short integuments 1* (*sin1*) mutants produce altered integuments. The *sup* mutation, initially identified by its effects on stamen number and carpel development (SCHULTZ *et al.* 1991; BOWMAN *et al.* 1992), also produces ovules in which the outer integument lacks the marked asymmetry found in WT ovules (GAISER *et al.* 1995). This results in ovules with a long tubular appearance (GAISER *et al.* 1995). The recent cloning of the *SUP* gene (SAKAI *et al.* 1995) has shown that it encodes a protein with properties of a transcription factor. This same study also showed that *SUP* mRNA is present only in the funiculus and not in the integuments, indicating an apparent non-cell autonomous mechanism for the role of this gene in integument development. *sin1* ovules have both inner and outer integuments, but both of these structures are shorter than those of wild-type ovules due to reduced cell expansion (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994).

Recent work in petunia indicates that two genes (*FBP7* and *FBP11*) encoding putative transcription factors may play a critical role in initiation of ovule development in this species (ANGENENT *et al.* 1995; COLOMBO *et al.* 1995). Transgenic suppression of these genes causes a loss of ovule identity (ANGENENT *et al.* 1995), and transgenic overexpression of *FBP11* results in formation of ectopic ovules (COLOMBO *et al.* 1995). Thus, these genes may be primary determinants of ovule identity in petunia and may have counterparts with similar function in Arabidopsis (ANGENENT and COLOMBO 1996).

While the phenotypes produced by the Arabidopsis mutations provide insight into the roles the genes play in regulation of ovule development, a complete understanding of the regulation of this entire process can only be achieved by studying the interactions and epistatic relationships of the different mutations. Herein we characterize the novel *inner no outer* (*ino*) mutation, which leads to specific loss of only the outer integument, and five new *ant* alleles, which provide new insight into the function of this gene. By combining this analysis with characterization of phenotypes of double mutant plants, and analysis of other known ovule mutations, we have assembled a preliminary model for the genetic regulation of ovule development.

MATERIALS AND METHODS

Plant material: Seeds were sown in a 1:1:1 mixture of perlite, vermiculite, and peat moss. Plants were grown under continuous fluorescent and incandescent illumination at 22–25° and fertilized weekly with a complete nutrient solution (KRANZ and KIRCHHEIM 1987). Once germinated, the plants were treated weekly with either malathion or orthene to control insects. Genetic crosses were performed as previously described (KRANZ and KIRCHHEIM 1987).

Mutant screen and allelic designations: M2 *Arabidopsis thaliana* ecotype Landsberg *erecta* plants, derived from ethyl methanesulfonate-mutagenized parent seeds (Lehle Seeds, Round Rock, TX), were screened for female sterility as previously described (ROBINSON-BEERS *et al.* 1992). The *ant-4* allele was a gift from DAPHNE PRUESS (University of Chicago) and derived from a similar screen of seed from ethyl nitrosourea mutagenized material. All isolates are known to be independent as they differ significantly in phenotype or derive from independent pools of mutant seed. During the course of this research, we learned that the laboratories of ROBERT FISCHER (University of California, Berkeley) and DAVID SMYTH (Monash University, Clayton, Victoria, Australia) had isolated mutants similar to ours that lacked integuments. Complementation tests showed these other isolates were allelic to our isolates. Together, we agreed to use the *ANT* designation for this locus and collaborated in assignment of specific allele numbers to the different isolates.

Genetic mapping: The *ANT* gene was mapped relative to the cleaved amplified polymorphic sequences (CAPS) markers DHS and AG (KONIECZNY and AUSUBEL 1993) on F_2 progeny (32 chromosomes analyzed for DHS, 36 chromosomes analyzed for AG) from an *ant-4/ant-4* Ler X Co-3 cross. The *INO* gene was mapped relative to the SSLP (simple sequence length polymorphism) markers nga63 and nga248 (BELL and ECKER 1994) on F_2 progeny (196 chromosomes analyzed) from an *ino/ino* Ler × Co-3 cross. Map distances in both cases were determined using the MAPMAKER computer program (LANDER *et al.* 1987).

Microscopy: Samples were prepared for scanning electron microscopy as previously described (ROBINSON-BEERS *et al.* 1992) and were examined on a ISI DS130 scanning electron microscope (Topcon Technologies, Paramus, NJ) at an accelerated voltage of 10 kV. The preparation and photography of sections of plastic embedded pistils was as previously described (ROBINSON-BEERS *et al.* 1992). For confocal laser scanning microscopy, Arabidopsis inflorescences were fixed and stained with fluorescent periodic acid-Schiff (PAS) reagent as described by VOLLBRECHT and HAKE (1995). For some samples, an additional aldehyde blocking step was performed by treating samples with a saturated aqueous solution of dimedone (5,5-dimethyl-1,3-cyclohexanedione) overnight at room temperature prior to staining. The samples were then stored at 4° until ready for further processing. Pistils from individual flowers or buds were then removed from cleared inflorescences and were placed on a slide in a small amount of methyl salicylate. Visibility of the cleared tissue was enhanced through the use of dark field optics to facilitate dissections.

Stained samples were examined and images were captured with a Zeiss LSM 410 invert Laser Scan microscope, software version 3.50. Excitation illumination was from an Ar/Kr laser emitting at 488 nm and a barrier filter passing 515–540 nm wavelengths was used to view the fluorescence. Image enhancement and false color addition were accomplished using

TABLE 1

Summary of *ant* and *ino* isolates

Mutant	Description	F_2 segregation		
		Observed	χ^{2a}	P^a
ant-4	Minimal chalazal bend; low ridge of cells	64:21 (3.05:1)	0.004	0.95
ant-5	Intermediate chalazal bend; ridge of cells	163:69 (2.36:1)	2.782	0.095
ant-6	Intermediate chalazal bend; ridge of cells	182:60 (3.10:1)	0.006	0.94
ant-7	Normal chalazal bend; expanded ridge of cells	116:36 (3.22:1)	0.140	0.71
ant-8	Normal chalazal bend; expanded ridge of cells	125:36 (3.47:1)	0.598	0.44
ino	Transient chalazal bend, single integument	146:43 (3.39:1)	0.510	0.48

[a] For expected 3:1, wild type:mutant.

NIH Image software, v. 1.58 (available at http://rsb.info.nih.gov/nih-image/).

Double mutant analysis: *ino bel1:* Homozygous *ino* plants were used to pollinate emasculated *bel1-1/BEL1* plants. Seed was collected from each of four F_1 plants, all of which exhibited a wild-type phenotype. Two resulting F_2 families segregated for both *bel1* and *ino* with a ratio of 545 wild type:258 $Bel1^-$:161 Ino^- plants. The independent segregation of these genes is assured as they reside on different chromosomes [chromosome *V* for *BEL1* (RAY *et al.* 1994), and chromosome *I* for *INO*]. The absence of a phenotypically novel class of plants leaves three possibilities for the double mutant: the double mutant could be lethal, have a $Bel1^-$ phenotype, or have an Ino^- phenotype, resulting in a 9:3:3, a 9:4:3, or a 9:3:4 ratio, respectively. The large number of plants analyzed allowed us to statistically reject the 9:3:3 hypothesis (χ^2 = 35.2, $P < 10^{-5}$) and the 9:3:4 hypothesis (χ^2 = 59.6, $P < 10^{-5}$), leaving only the 9:4:3 hypothesis (χ^2 = 3.371, P = 0.185). This indicates that the double mutant has a $Bel1^-$ phenotype. Because of the complete female sterility of both mutants, direct testing for the double mutant genotype through crossing to homozygous single mutant plants is not possible. Crosses of putative double mutants to heterozygous plants requires statistical analysis with no advantage over the direct analysis of the F_2 generation.

ino sin1: Homozygous *ino* plants were used to pollinate emasculated *sin1-1/SIN1* plants. F_2 seed was collected from each of eight phenotypically wild-type plants. A novel phenotypic class was observed among the resultant F_2 population, which segregated 103 wild type:30 $Sin1^-$:47 Ino^-:3 $Sin1^-$ Ino^- plants, which did not fit a 9:3:3:1 ratio (χ^2 = 11.458, P = 0.00949). However, *sin1* is known to segregate at a lower ratio than 3:1 (~4:1) when planted in soil (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994). The data more closely fit the 12:3:4:1 ratio (χ^2 = 7.7468, P = 0.05154) predicted for the aberrant segregation of *sin1*.

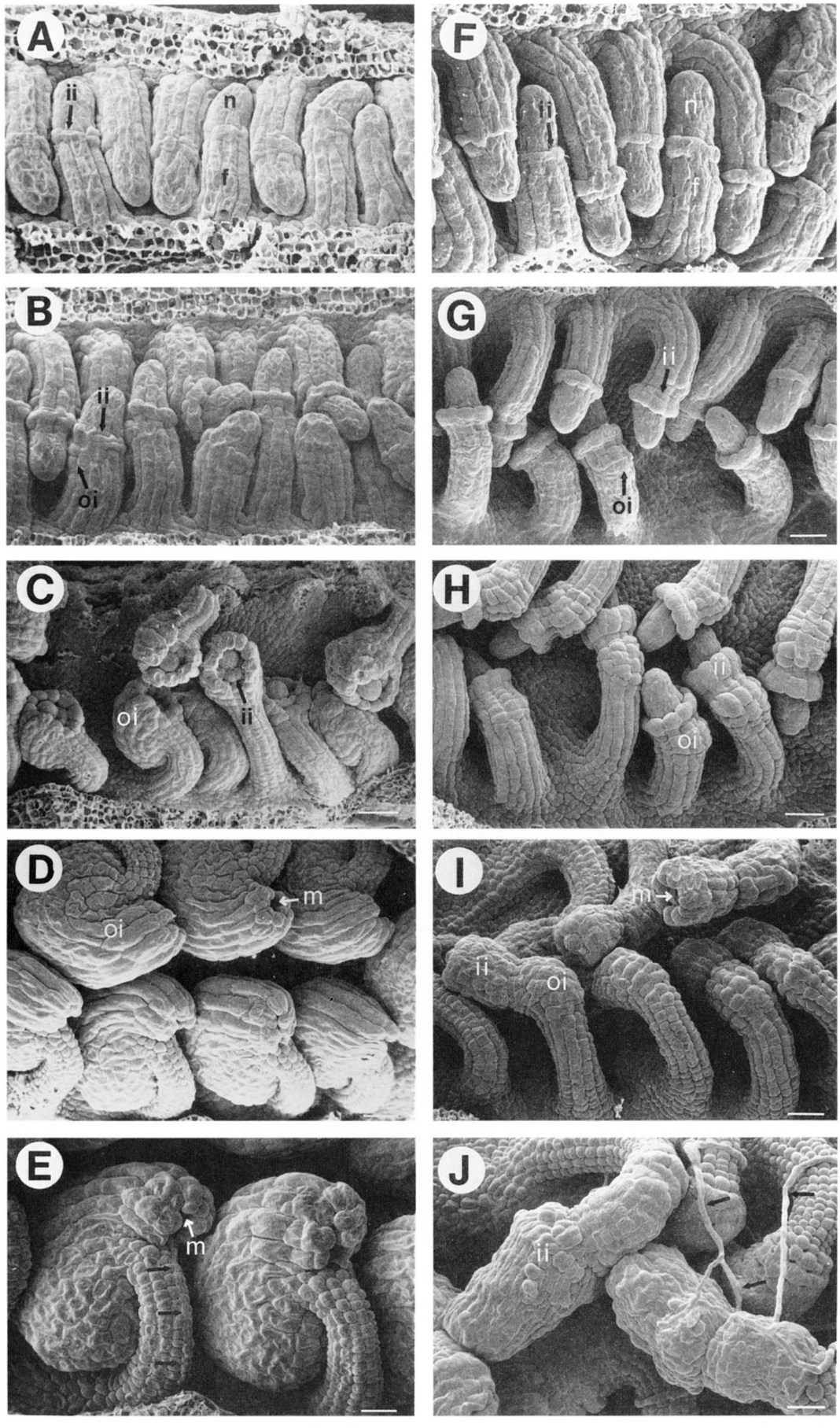
ino ats: Homozygous *ino* plants were used to pollinate homozygous *ats* plants. F_2 seed were collected from each of two phenotypically normal F_1 plants, both of which segregated for both mutations. A novel phenotypic class was observed among the resulting F_2 population, which segregated 62 wild type:21 Ino^-:18 Ats^-:6 Ino^- Ats^- (see RESULTS). These data fit the expected 9:3:3:1 ratio (χ^2 = 0.381, P = 0.94).

ant bel1: Plants homozygous for *ant-4* were crossed with plants heterozygous for *bel1-1*, and plants homozygous for *bel1-1* were crossed with plants heterozygous for either *ant-5* or *ant-6*. Progeny of 18 of 28 F_1 plants (all of which were phenotypically wild type) showed segregation for both *ant* and *bel1*. F_2 results were similar for all three alleles of *ant*, allowing us to combine the data sets for statistical analysis. The combined F_2 segregated 469 wild type:213 Ant^-:187 $Bel1^-$ plants. The independent segregation of these genes is assured as they reside on different chromosomes [chromosome *V* for *BEL1* (RAY *et al.* 1994), and chromosome *IV* for *ANT*]. The absence of a novel phenotype for the *ant bel1* double mutant leaves three possibilities for the double mutant phenotype; lethal, Ant^-, or $Bel1^-$, which would result in 9:3:3, 9:4:3 and 9:3:4 ratios, respectively. We were able to statistically exclude the 9:3:3 (χ^2 = 19.7, $P = 5 \times 10^{-5}$) and 9:3:4 (χ^2 = 20.4, $P = 4 \times 10^{-5}$) ratios, but not the 9:4:3 ratio (χ^2 = 4.44, P = 0.11), indicating an Ant^- phenotype for the double mutant. As mentioned above, direct testing for the genotype of the putative double mutants was not possible due to the complete female infertility of both mutants.

ant sin1: Homozygous *ant-4* plants were used to pollinate plants heterozygous for *sin1*. All F_1 plants were phenotypically wild type. Progeny of two of five F_1 plants (all of which were phenotypically wild type) showed segregation for both *ant-4* and *sin1*. Families including both $Ant\text{-}4^-$ and $Sin1^-$ plants also segregated for plants with the $Sin1^-$ vegetative phenotype and $Ant\text{-}4^-$ ovules. The segregation ratios in these families (51 wild type:10 Ant^-:7 $Sin1^-$:2 Ant^- $Sin1^-$) did not conform to a 9:3:3:1 ratio (χ^2 = 8.324; P = 0.04) but did conform to the 12:4:3:1 ratio predicted for this cross (χ^2 = 4.881, P = 0.18) (see above).

ant sup: Homozygous *sup-5* plants were used to pollinate plants heterozygous for *ant-5*. All F_1 plants were phenotypically wild type. Progeny from the subset of F_1 plants that showed segregation for both *ant-5* and *sup-5* in the F_2 generation (two of three F_2 families examined) were further analyzed. These families also included plants with $Sup\text{-}5^-$ flowers and $Ant\text{-}5^-$ ovules, and the frequencies of all phenotypes (84 wild type:30 Ant^-: 25 $Sup\text{-}5^-$:6 Ant^- $Sup\text{-}5^-$) were consistent with the expected 9:3:3:1 ratio (χ^2 = 1.575, P = 0.67).

ant ino: Homozygous *ino* plants were used to pollinate plants heterozygous for *ant-5*. All F_1 plants were phenotypically wild type. Progeny from the subset of F_1 plants that showed segregation for both *ant-5* and *ino* in the F_2 generation (two of five F_2 families examined) were further analyzed. The independent segregation of these genes is assured as they reside on different chromosomes (chromosome *I* for *INO*, and chromosome *IV* for *ANT*). A novel phenotype for the *ant ino* double mutant was not found, leaving three possibilities for the double mutant phenotype; lethal, Ant^-, or Ino^-, which would result in 9:3:3, 9:4:3 and 9:3:4 ratios, respectively. We were unable to statistically exclude any of the possibilities; 9:3:3 (χ^2 = 5.2, P = 0.07), 9:4:3 (χ^2 = 5.0, P = 0.08) and 9:3:4 (χ^2 = 1.3, P = 0.53). From this population we selected 10 phenotypically Ant^- plants (the F_0' generation) and backcrossed these plants to wild-type Ler plants as a first step in evaluating their genotype at the *INO* locus. Approximately 10 F_1' plants from each cross were planted and, as expected, all were phenotypically wild type. These plants were allowed to self-fertilize, and seeds were collected from each. F_2' plants


A
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were then scored for the Ant⁻ and Ino⁻ phenotypes. All F_2' populations from all F_1' parents segregated for the *ant* mutation, indicating that, as expected, each F_1' plant was heterozygous for *ant* because each F_0' plant was homozygous for *ant*. In addition, for two of the F_0' plants, *all* F_1' plants (nine of nine and 13 of 13) produced F_2' populations segregating for *ino*. χ^2 analysis shows that the probabilities that these two F_0' plants were not homozygous for *ino* was 0.0027 and 0.00031, respectively. Therefore, these two phenotypically Ant⁻ F_0' plants were homozygous for both *ant* and *ino*, demonstrating an Ant⁻ phenotype for the double mutant.

RESULTS

Isolation of *ant* and *ino* mutants: We have previously described the isolation of Arabidopsis ovule mutants through screening a mutagenized population for reduced female fertility (ROBINSON-BEERS *et al.* 1992). In a continuation of this screen, five new isolates were found that had ovules with reduced or absent integuments. Through direct complementation tests these mutants [originally referred to as *dragon* mutants, (GASSER *et al.* 1994)] were found to represent new alleles of a locus now termed *aintegumenta* (*ant*) (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996). Another isolate, with ovules lacking only the outer integuments, represented a different locus termed *inner no outer* (*ino*) (GAISER *et al.* 1995). Table 1 summarizes the phenotypic effects of each of these mutations. The five *ant* isolates constitute an allelic series displaying a range in the severity of effects on integument formation, but all five exhibit complete female sterility. In contrast, the *ino* mutant produced a very small number of aberrant but viable seeds. Plants homozygous for *ant* or *ino* mutations were vegetatively normal, other than exhibiting general effects associated with infertility. All five *ant* alleles and the *ino* mutation showed segregation ratios very close to 3:1 (Table 1), indicating that they represent single recessive loci.

The *ANT* gene was mapped to chromosome *IV*, showing 0% recombination with molecular marker DHS1 out of 32 chromosomes examined and 19% recombination with *AG* out of 36 chromosomes examined. The *INO* gene mapped to chromosome *I*, showing 13.5 and 6.2% recombination with molecular markers nga63 and nga248 (BELL and ECKER 1994), respectively (196 chromosomes examined).

Wild-type ovule development: Figure 1 shows scanning electron micrographs of representative stages of wild-type and *ino* ovule development. Arabidopsis ovule development takes place during stages 8–14 of flower development (flower development stages from SMYTH *et al.* 1990) and has been previously described (ROBINSON-BEERS *et al.* 1992; MODRUSAN *et al.* 1994; SCHNEITZ *et al.* 1995). SCHNEITZ *et al.* (1995) recently proposed a nomenclature for the stages of ovule development, and this system will be used in our descriptions. During ovule stage 1, finger-like ovule primordia initiate from the placental tissue in the ovary. In stage 2-II, the inner integument is initiated through division and expansion of a symmetrical ring of cells at the chalaza, demarcating the terminal nucellus and supporting funiculus (Figure 1A). In stage 2-III, the outer integument initiates as an asymmetrical wedge of cells proximal to the inner integument (Figure 1B). The wider part of this integument primordium is characteristically oriented toward the base of the ovary and toward the septum separating the two locules of the ovary. For ease of description, we refer to this as the abaxial side of a developing ovule. During stages 3 and 4, the inner integument expands symmetrically, enveloping the nucellus. The outer integument expands asymmetrically and covers the inner integument as a result of extensive cell division, cell elongation on the abaxial side of the primordium, and minimal growth on the opposite or adaxial side (Figure 1, C–E). Throughout this process, asymmetrical expansion of the funiculus leads to a marked curvature of this structure (Figure 1, B and C) in the abaxial direction. In addition, a progressive bending occurs at the chalaza in a direction opposite to the curvature of the funiculus resulting in an overall S-shape of the ovules (Figure 1, A–E). During stage 2, meiosis occurs within the nucellus (SCHNEITZ *et al.* 1995), and during stage 3, one of the resulting megaspores goes on to form the embryo sac (visible in Figure 2A).

***ino* ovule morphogenesis:** In early development, *ino* ovules exhibit the same abaxial curvature toward the septum and base of the ovary observed in wild-type ovules (Figure 1, F and G). Initiation of the outer integument (at stage 2-III) marks the onset of deviation from wild-type development. The asymmetric form of the Ino⁻ outer integument primordium resembles that of a wild-type ovule; however, the wider region of the outer integument primordium in the mutant is oriented away from the septum and toward the top of the ovary on the adaxial side of the ovule primordium (Figure 1, G

FIGURE 1.—Scanning electron micrographs of ovule development in wild-type arabidopsis and *ino* mutants. In all panels the base of the ovary (and hence, the abaxial side of the ovule primordia) is to the left. (A) Wild-type ovules at stage 2-II. The inner integument has initiated and separates the nucellus and funiculus. Bar, 18 μm. (B) Wild-type ovules at stage 2-III. The outer integument has initiated on the abaxial side of the ovule primordia. The funiculus has begun to curve in the abaxial direction and the nucellus has begun to bend in the adaxial direction. Bar, 25 μm. (C) Wild-type ovules at stage 3-I. Bar, 25 μm. (D) Wild-type ovules at stage 4-I (anthesis). Bar, 25 μm. (E) Wild-type ovules at stage 4-I (anthesis); a pollen tube (arrows) is visible entering the micropyle of one ovule. Bar, 17 μm. (F) Ino⁻ ovules at stage 2-II. Bar, 12 μm. (G) Ino⁻ ovules at stage 2-III. The outer integument primordia are initiating on the adaxial sides of the ovule primordia. Bar, 20 μm. (H) Ino⁻ ovules at stage 3-I. Bar, 20 μm. (I) Ino⁻ ovules at stage 4-I. The inner integument has covered the nucellus; micropyles face the base of the ovary. Bar, 25 μm. (J) Ino⁻ ovules at stage 4-I. Pollen tubes (arrows) grow apparently at random. Bar, 17 μm. f, funiculus; ii, inner integument; m, micropyle; n, nucellus; oi, outer integument.

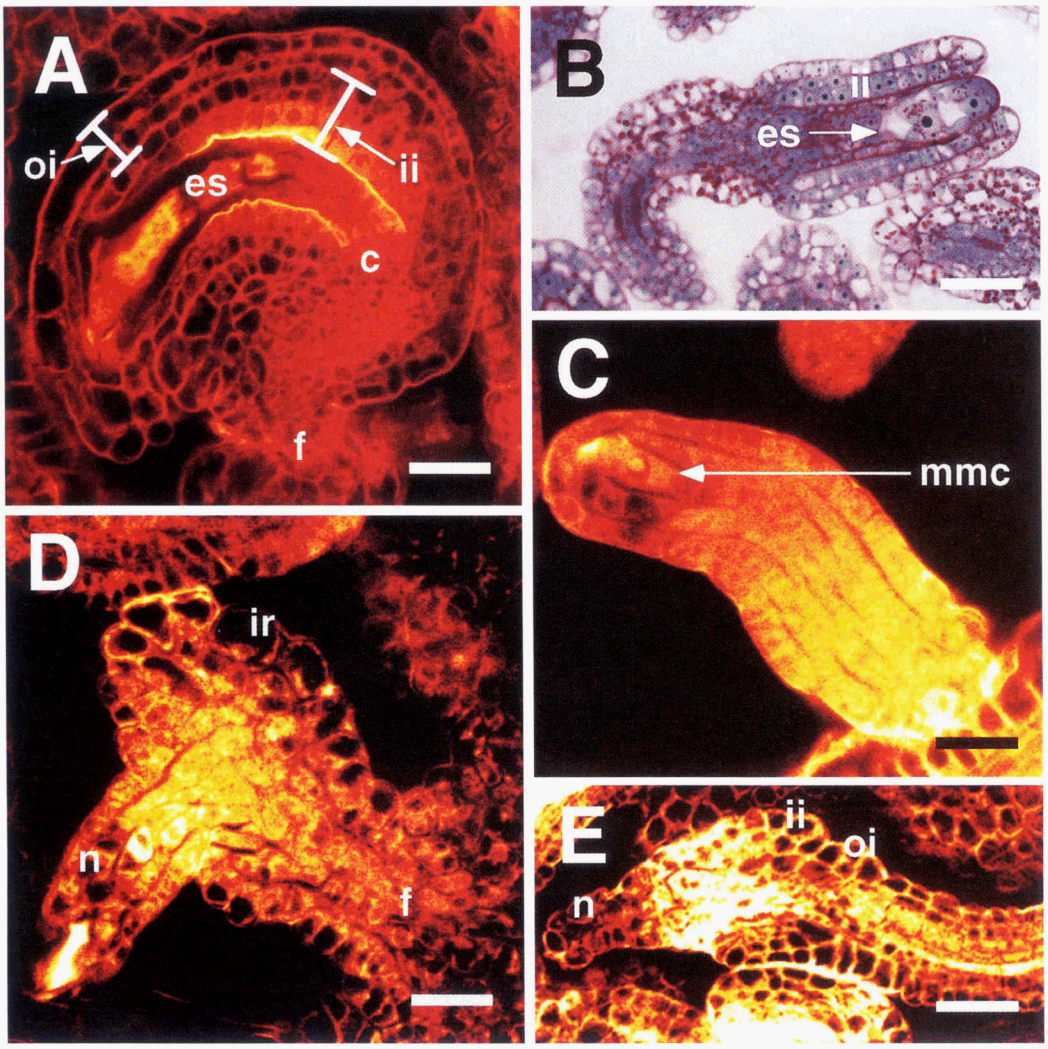

FIGURE 2.—Anatomy of wild-type, Ant$^-$ and Ino$^-$ ovules. All panels are confocal sections of ovules stained with PAS, except as noted. (A) Wild-type ovule at stage 4-I. Bar, 30 μm. (B) Plastic embedded section of Ino$^-$ ovule at stage 4-I. Bar, 20 μm. (C) Ant-4$^-$ ovule at stage 4-I. The integumentary ridge is not evident in this section. Bar, 10 μm. (D) Ant-8$^-$ ovule at stage 4-I. Bar, 15 μm. (E) Ino$^-$ Ats$^-$ double mutant ovule at stage 4-I. Bar, 30 μm. c, chalaza; es, embryo sac; f, funiculus; ii, inner integument; ir, integumentary ridge; mmc, megaspore mother cell; n, nucellus; oi, outer integument.

and H). Thus, initiation of the outer integument of an Ino$^-$ ovule occurs at a position ~180° rotated from, or opposite, the normal site of initiation. Despite the misorientation of the outer integument primordium there appears to be normal initiation of adaxial bending at the chalaza (Figure 1, G and H).

Further development of the inner integument of an Ino$^-$ ovule appears to proceed normally with anticlinal divisions of the primordial cells producing a sleeve-like inner integument, which covers the nucellus (Figure 1I). In contrast, the outer integument undergoes little additional development, remains rudimentary, and fails to cover the inner integument (Figure 1, I and J). At anthesis, only the inner integument covers the nucellus. Furthermore, apparently as a result of the absence of an outer integument, bending at the chalaza does not progress as far as in wild-type ovules, and the overall shape of Ino$^-$ ovules is that of a C rather than the S shape seen in wild-type ovules (compare Figure 1, I with C and D). Thus the micropyle (the opening in the integuments) of Ino$^-$ ovules is not adjacent to the funiculus but is essentially along a line from the funiculus through the nucellus. Taking all features into account, Ino$^-$ ovules can be classified as unitegmic and orthotropous.

After anthesis and pollination, the behavior of pollen tubes within the Ino$^-$ pistils also differs from that of wild type. Pollen tube growth occurs in an apparently random fashion from the septum to the surface of the ovules (Figure 1J). In contrast, in wild-type pistils pollen tubes typically make their way from the septum to the micropyle of the ovule along the funiculus (Figure 1E and see HULSKAMP *et al.* 1995).

Figure 2B illustrates the internal anatomy of an Ino$^-$ ovule. Like the inner integuments of wild-type ovules at the time of anthesis (SCHNEITZ *et al.* 1995), those of Ino$^-$ ovules consist of two cell layers, except near the chalaza where three layers occur. The inner integuments of Ino$^-$ ovules were less curved than those of wild-

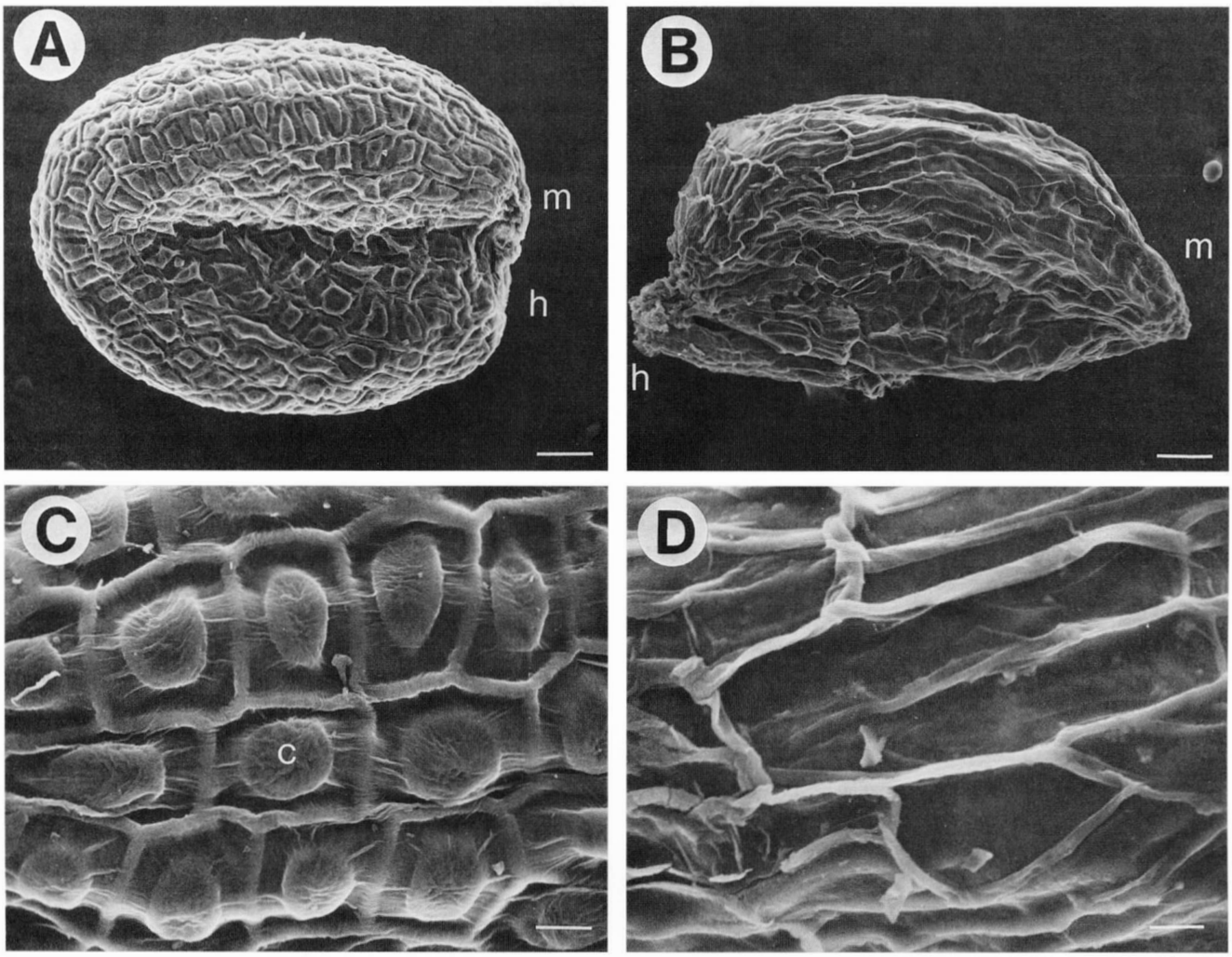

FIGURE 3.—Scanning electron micrographs of wild-type and Ino$^-$ seeds. (A) Wild-type seed. Bar, 45 μm. (B) Ino$^-$ seed. Bar, 45 μm. (C) Surface features of wild-type seed. Bar, 10 μm. (D) Surface features of Ino$^-$ seed. Bar, 10 μm. m, micropyle; h, hilum; c, columella.

type ovules. Within the nucellus an apparently normal embryo sac is formed.

Abnormal features of Ino$^-$ seeds: The *ino* mutation dramatically reduced female fertility with each homozygous *ino* plant producing approximately one to three seeds. The abnormal shape of such seeds is illustrated in Figure 3. Wild-type ovules exhibit amphitropous morphology, where the funiculus attaches in close proximity to the micropyle (Figure 1E). As a result, the hilum (the scar left following detachment from the funiculus) is adjacent to the micropyle in wild-type seed (Figure 3A). The orthotropous form of Ino$^-$ ovules leads to the positioning of the micropyle and hilum at opposite ends of the seeds of *ino* plants (Figure 3B). Embryos formed within Ino$^-$ ovules appear normal in outline beneath the seed coat. We have not specifically measured the dormancy period required for efficient germination of Ino$^-$ seeds. However, we do observe a significant frequency of germination of undessicated seeds while they are still in the siliques of parental plants (vivipary), indicating that these seeds exhibit reduced dormancy.

The outer surfaces of Ino$^-$ seeds also differ from those of wild-type seeds (compare Figure 3, C and D). Whereas the seed coat of a wild-type seed comprises both the inner integument (tegmen) and outer integument (testa), that of an Ino$^-$ seed derives only from the inner integument. The outer surface of a wild-type seed coat consists of relatively isodiametric, polygonal cells with central elevations (the columellae) (Figure 3C). By contrast, the outer surface of the seed coat of an Ino$^-$ seed is made up of elongated cells that lack columellae (Figure 3D). The normal yellow-brown color of Arabidopsis seeds results from pigmentation of the inner layers of the tegmen. Ino$^-$ seeds were normal in color, indicating that this aspect of differentiation of the inner integument into the tegmen was normal.

***ant* ovule morphogenesis:** Plants homozygous for any of the five *ant* alleles we have isolated exhibited incomplete integument development. As illustrated in Figure 4, however, the degree to which the integuments develop varied among the different alleles. In the strongest *ant* allele (the allele with the greatest deviation from wild type, *ant-4*), ovule primordia form and develop

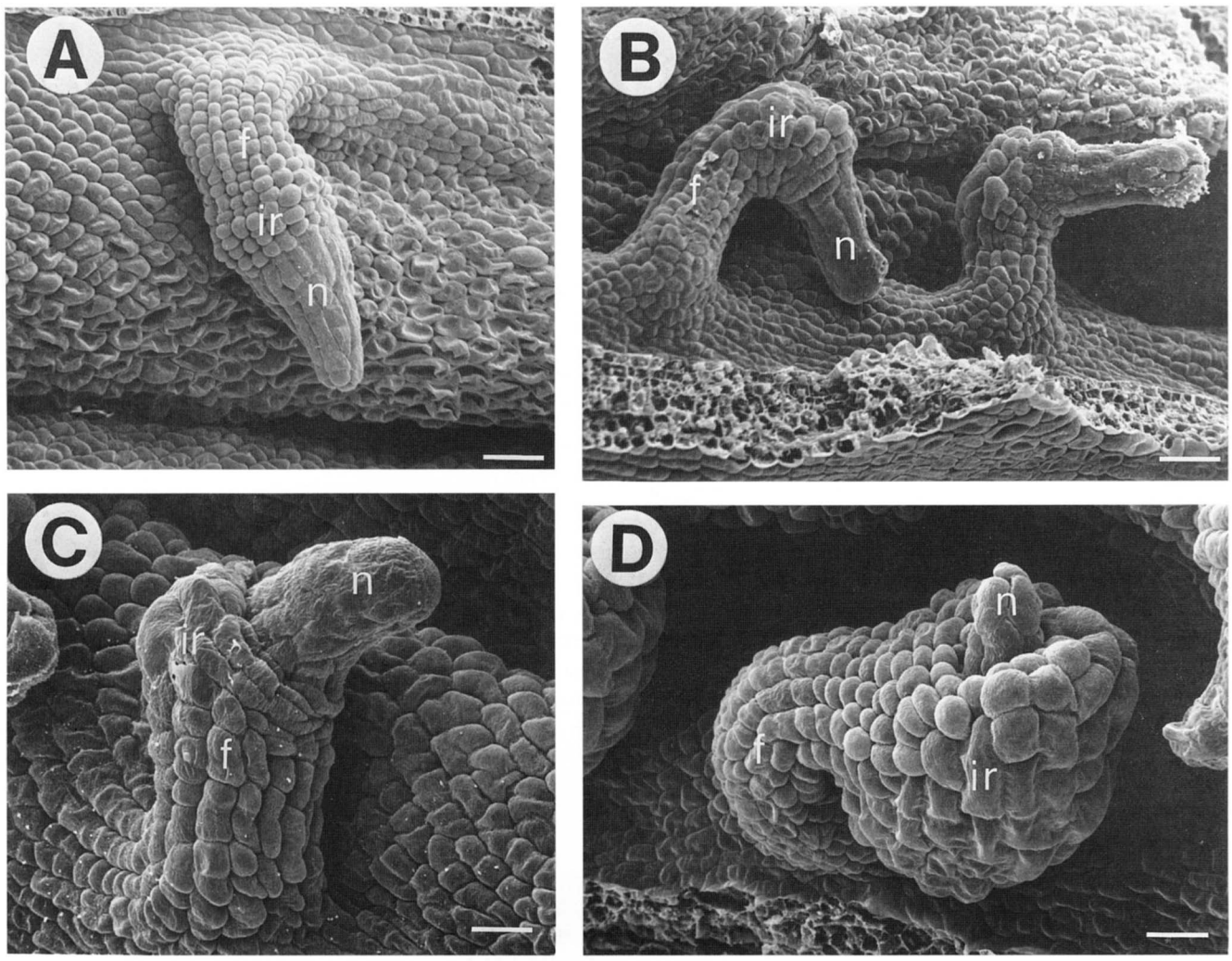

FIGURE 4.—Scanning electron micrographs of Ant$^-$ mutant ovules. (A) Ant-4$^-$ ovule at stage 4-I. The cells of the nucellus and funiculus are clearly differentiated from one another. Bar, 20 μm. (B) Ant-5$^-$ ovule at stage 4-I. The integumentary ridge of cells is larger than that of Ant-4$^-$ ovule. Bar, 20 μm. (C) Ant-8$^-$ ovule at late stage 2. Bar, 10 μm. (D) Ant-8$^-$ ovule at stage 4-I. The integumentary ridge of cells has expanded to almost cover the nucellus. Bar, 14 μm. f, funiculus; ir, integumentary ridge; n, nucellus.

normally through stage 2-I (data not shown). During stages 2-II and 2-III, in place of the two integument primordia, only a slightly elevated ridge of cells, the "integumentary ridge," forms at the chalaza of *ant-4* ovules (Figure 4A). The integumentary ridge demarcates the nucellus and the funiculus but undergoes no further expansion. Development of other parts of the ovule does not immediately arrest, however, as the effects of subsequent differentiation of the surface cells of the nucellus and funiculus are clearly visible at anthesis (Figure 4A). In a slightly weaker allele (*ant-5*), the ridge of cells undergoes limited asymmetrical expansion, and the ovules take on more of the characteristic S shape of wild-type ovules (Figure 4B). In the weakest allele (*ant-8*), the integumentary ridge expands further and, in some cases, partially surrounds the nucellus and superficially resembles an outer integument (Figure 4, C and D). While the ovules pictured in Figure 4 are typical of the different *ant* alleles, there is significant variability among individual ovules in plants homozygous for each allele. There is some overlap in phenotypes observed for alleles that are adjacent to each other in the allelic series. For example, the most severely affected ovules of *ant-5* mutants are similar to the least affected ovules of *ant-4* mutants. There is, however, essentially no overlap between phenotypes of the strongest and weakest alleles.

***ant* ovule anatomy:** To determine the effects of *ant* mutations on embryo sac development and to reveal further details of aberrations in integument development, confocal laser scanning microscopy was used to examine serial optical sections of >400 Ant$^-$ ovules. In the vast majority of these sections, we observed a single, enlarged cell, clearly differentiated from the surrounding tissue, near the tip of the nucellus (Figure 2C). A megaspore tetrad was never observed. Together, these observations led us to interpret the differentiated cell as being a megaspore mother cell, and to conclude that meiosis did not occur in any of our *ant* mutants.

Analysis of the weakest allele, *ant-8*, showed from

which cells of the chalaza the integumentary ridge arose (Figure 2D). Comparison of Ant-8$^-$ ovules with wild-type ovules of similar size (see Figure 1B, for example) revealed that the number of cells giving rise to the integumentary ridge of Ant-8$^-$ ovules, and the two integuments of wild-type ovules was roughly equivalent. Therefore, at least in the weakest *ant* allele, the integumentary ridge derives from cells that would give rise to the two integuments in wild-type ovules.

Effects of *ant* on other floral structures: Flowers of homozygous *ant* plants exhibit other abnormalities in addition to aberrant ovule development. Ant$^-$ petals are narrow and have somewhat irregular margins when compared with wild-type petals (compare Figure 5, A and B). No variations were seen among the petal phenotypes of the different *ant* alleles, indicating that petal shape is less affected by variations in the alleles than are ovule phenotypes. Ant$^-$ flowers have a variable, but generally reduced, number of stamens. Flowers with four, five, or six stamens were commonly observed, and the mean number of stamens in a count of 110 flowers was 4.6 (*vs.* 5.75 for 101 wild-type flowers). The reduction in stamen number resulted specifically from loss of lateral stamens as four medial stamens were invariably present. All six nectaries were present in most mutant flowers; however, in a small number of cases (six of 110 flowers) absence of a lateral stamen was accompanied by absence of the associated lateral nectary.

Pistils of *ant* plants showed slight but consistent developmental aberrations. The stigmas of Ant$^-$ pistils were somewhat smaller in size and consisted of a smaller number of stigmatic cells than did the stigmas of wild-type pistils (compare Figure 5, E and F).

Genetic analysis: Double mutant analysis was performed with *ant, ino* and other known ovule development mutations to assess genetic interactions. Appropriate crosses were made to generate populations that segregated for both mutations being studied and that would include plants homozygous for both mutant genes. Representative single and double mutants are illustrated in Figure 6.

ino bel1: Ovules of plants homozygous for *bel1* mutations initiate only a single asymmetrical integument-like structure (ILS) in place of the two integuments of wild-type ovules (ROBINSON-BEERS *et al.* 1992; MODRUSAN *et al.* 1994). In the case of the *bel1-1* allele, the ILS forms an amorphous collar of tissue that surrounds, but does not cover, the nucellus (ROBINSON-BEERS *et al.* 1992).

A population of 964 plants segregating for both *ino* and *bel1-1* included only wild-type plants, Ino$^-$ plants, and Bel1$^-$ plants. The independent segregation of these genes is assured as they reside on different chromosomes [chromosome *V* for *BEL1* (RAY *et al.* 1994) and chromosome *I* for *INO*]. We thought it possible that the earliest aberrations in integument primordium formation resulting from the *ino* mutation could occur before the first effects of the *bel1-1* mutation and result in a novel phenotype. Close examination of the Bel1$^-$ plants failed to reveal a subset with a clear consistent difference from other members of this class. Statistical analysis of the data (see MATERIALS AND METHODS) demonstrates that we can reject all hypotheses except that of the double mutant being indistinguishable from a *bel1-1* single mutant. This indicates that *bel1-1* is epistatic to *ino*.

ino sin1: In the Landsberg erecta background, plants homozygous for *sin1* form ovules with short integuments and an exposed nucellus (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994) (Figure 6A). In addition to this ovule phenotype, Sin1$^-$ plants exhibit a number of pleiotropic effects including small leaves, short internodes, and stamens that mature later and produce significantly less pollen than those of wild-type plants (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994).

Three plants with the Sin1$^-$ vegetative phenotype, but with ovules that differed significantly from Sin1$^-$ ovules, were identified in a population segregating for both *sin1* and *ino*. Like the ovules of *ino* single mutants, ovules of these plants had aberrantly oriented, abortive outer integuments (Figure 6B). However, in contrast to *ino* single mutant ovules, the inner integuments of these ovules did not fully expand and failed to cover the nucelli. Thus these plants exhibited a combination of features of both Ino$^-$ and Sin1$^-$ phenotypes. This, together with their frequency in the population (see MATERIALS AND METHODS), indicated that these plants were *sin1 ino* double mutants. Thus, the effects of the *sin1* and *ino* mutations appear to be largely additive.

ino ats: Ovules of plants homozygous for *ats* produce only a single integument in place of the two normal integuments (Figure 6D) (LEON-KLOOSTERZIEL *et al.* 1994). On the basis of cell layer numbers and internal anatomy, it has been hypothesized that the single integument of Ats$^-$ ovules results from a fusion of the inner and outer integuments (LEON-KLOOSTERZIEL *et al.* 1994). Ats$^-$ ovules include normal embryo sacs, are fertile, and develop into aberrantly shaped seeds (LEON-KLOOSTERZIEL *et al.* 1994).

Plants with a novel ovule phenotype were identified in a population segregating for both *ats* and *ino*. Ovules of these plants exhibited the C-shape characteristic of Ino$^-$ ovules, but separation of the aberrantly oriented outer integument primordium and the adjacent inner integument primordium was incomplete (Figure 6, E and F). Further development of the inner integument consisted primarily of lateral expansion and the normal sheath-like character of the inner integument was never observed. Rather, only a thickened region of tissue, contiguous with the abortive outer integument, was formed in place of the inner integument (Figure 6, E and F). Both the phenotype and frequency of plants with the novel ovule phenotype were consistent with their being *ino ats* double mutants (see MATERIALS AND METHODS). In contrast to both *ats* and *ino* single mutants, which produce normal embryo sacs, the nucelli of *ats ino* double mutant ovules included single enlarged cells with

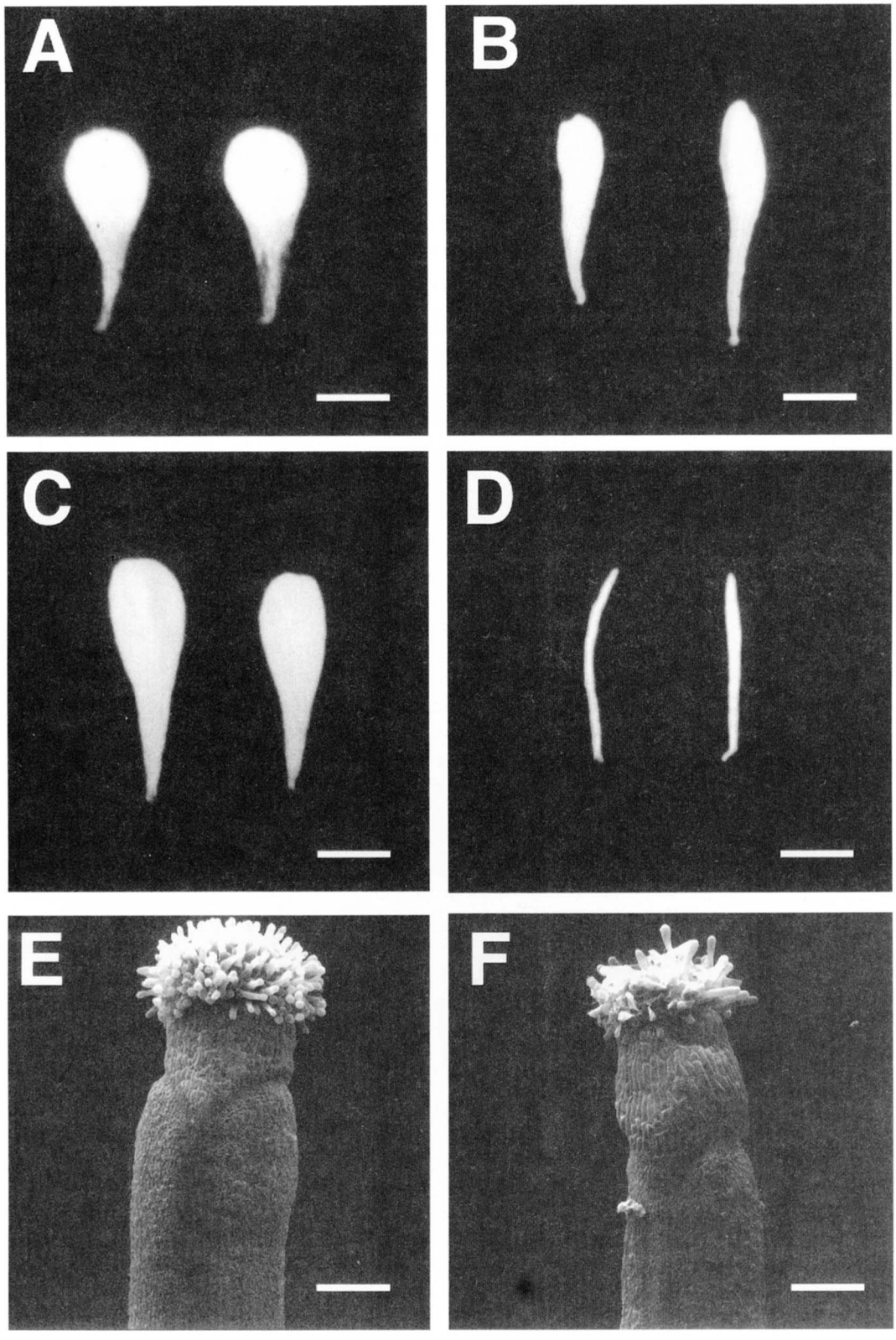

FIGURE 5.—Other floral effects of *ant*. (A) Wild-type petals. Bar, 1.3 mm. (B) Ant$^-$ petals. Narrow petals with irregular margins. Bar, 1.3 mm. (C) Sin1$^-$ petals. These petals are slightly narrower than wild type. Bar, 1.3 mm. (D) Ant$^-$ Sin1$^-$ petals. Bar, 1.3 mm. (E) Wild-type pistil. Bar, 150 μm. (F) Ant$^-$ pistil. Somewhat smaller stigma with fewer cells than wild type. Bar, 150 μm.

no evidence of further embryo sac development (Figure 2E). Thus, while neither *ino* nor *ats* mutations prevents embryo sac formation, the combination of the two lead to the loss of this structure.

ant bel1: Of 869 plants segregating for both *ant* (either *ant-4*, *-5*, or *-6*) and *bel1-1*, no plants with novel phenotypes were observed. Statistical analysis showed significant overrepresentation of the Ant$^-$ phenotype in the segregating population, and this overrepresentation was consistent only with an Ant$^-$ phenotype for the double mutant (see MATERIALS AND METHODS). We conclude, therefore, that the double mutants were indistinguishable from *ant* mutants and that the tested alleles of *ant* were epistatic to *bel1-1*.

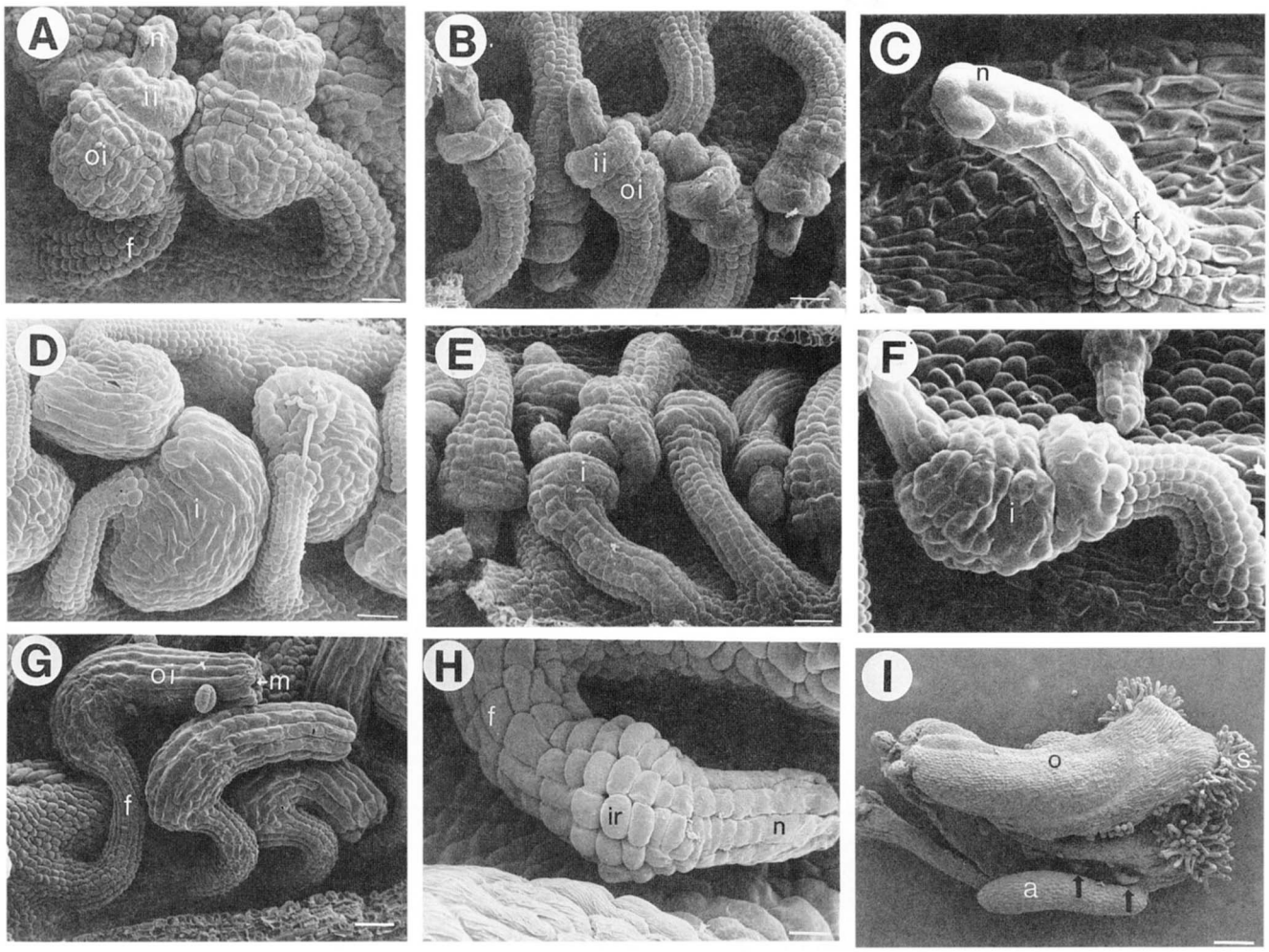

FIGURE 6.—Scanning electron micrographs of representative double mutants. (A) Sin1$^-$ ovules at stage 4-I. Bar, 17 μm. (B) Ovules of an *ino sin1* double mutant at stage 4-I. Bar, 22 μm. (C) Ovule of an *ant-4 sin1* double mutant at stage 4-I displaying the Ant$^-$ phenotype. This particular ovule does not have a prominent integumentary ridge. Bar, 10 μm. (D) Ats$^-$ ovules at stage 4-I. The single integument covers the nucellus and a pollen tube is visible entering a micropyle. Bar, 28 μm. (E) Ovules of an *ino ats* double mutant at stage 4-I. Bar, 22 μm. (F) Ovule of an *ino ats* double mutant at stage 4-I. Bar, 14 μm. (G) Sup1–5$^-$ ovules at stage 4-I. Note the reduced asymmetry of the outer integument relative to wild-type ovules (Figure 1B). Bar, 30 μm. (H) Ovule of an *ant-5 sup-5* double mutant at stage 4-I displaying Ant-5$^-$ phenotype. Bar, 10 μm. (I) Pistil of an *ant-5 sup-5* double mutant at stage 4-I displaying the Sup-5$^-$ phenotype. Ant-4$^-$ ovules can be seen emerging from the ovary wall (arrows). Bar, 138 μm. a, anther-like structure; f, funiculus; ii, inner integument; ir, integumentary ridge; m, micropyle; n, nucellus; o, ovary wall; oi, outer integument; s, stigmatic tissue; i, integument (of *ats* mutant).

ant sin1: Two plants displaying the novel phenotypic combination of having Ant$^-$ ovules and Sin1$^-$ vegetative characteristics were identified from among 70 plants segregating for *ant-4* and *sin1*. On the basis of these characteristics and the frequency of these plants in the population (see MATERIALS AND METHODS), we conclude that these plants represent *ant-4 sin1* double mutants. Ovules of these plants could not be distinguished from those of *ant-4* single mutants (Figure 6C), and we therefore conclude that *ant* is epistatic to *sin1* with respect to integument development.

The *ant-4 sin1* double mutant plants displayed additional novel floral abnormalities. In flowers of these plants second whorl organs were reduced in width, commonly being tendril-like structures approximately equal in length to normal petals (Figure 5D). The cells on the surface of these organs, however, were similar to those of wild-type petals at the level of resolution of the scanning electron microscope (data not shown). *sin1* single mutants have not previously been reported to have aberrant petals (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994). Direct comparison of petals from homozygous *sin1* mutants to those from wild-type plants reveals a subtle difference in width (compare Figure 5, C with A). However, further analysis will be necessary to conclusively determine if the shapes of the Sin1$^-$ petals actually fall outside the range of variation of wild-type petals. As seen in *ant* single mutants, lateral stamens were commonly absent in flowers of these plants. However, the anthers of the stamens that were present usually failed to dehisce and contained even less pollen than those of *sin1* single mutants.

ant sup: In *sup* mutant plants, additional stamen tissue is produced at the expense of carpel tissue leading

to the presence of extra stamens, stamens fused to regions of carpelloid tissue (when present), and carpels (when present) that often are not closed (SCHULTZ *et al.* 1991; BOWMAN *et al.* 1992). Sup$^-$ ovules have long, tubular outer integuments due to a decrease in the asymmetrical growth pattern characteristic of wild-type outer integuments (GAISER *et al.* 1995) (Figure 6G).

A population segregating both for *ant-5* and *sup-5* mutations included plants with the increased stamen number and reduced carpel tissue characteristic of homozygous *sup* mutants (Figure 6I) and plants that also produce phenotypically Ant$^-$ ovules (Figure 6H) and Ant$^-$ petals (not shown). Based on this phenotype and statistical analysis (see MATERIALS AND METHODS), these plants were identified as being homozygous for both *ant-5* and *sup-5*. These plants show that *ant-5* is epistatic to *sup-5* with respect to ovule development and that *ANT* and *SUP* act in relative independence in their effects on other floral organs.

ino ant: A population segregating for both *ino* and *ant-5* included only plants with the wild-type, Ino$^-$, and Ant-5$^-$ phenotypes. The ratios between these phenotypes were such that we were unable to rule out either Ino$^-$ or Ant-5$^-$ as the double mutant phenotype. Therefore, 10 phenotypically Ant-5$^-$ plants were tested genetically for the presence of the mutant *ino* gene. Two of these plants were shown to be homozygous for both *ant* and *ino* mutations (see MATERIALS AND METHODS), demonstrating that *ant-5* is epistatic to *ino*.

DISCUSSION

Effects and genetic interactions of the *ino* mutation: It has been previously noted that a critical early step in ovule development is the separation of the ovule primordium along the proximal/distal axis into at least three distinct pattern elements the funiculus, chalaza and nucellus (REISER *et al.* 1995; SCHNEITZ *et al.* 1995). The phenotype of the *ino* mutant indicates that patterning in the ovule primordium is even more complex. First, the *ino* mutation affects only the outer integument within the chalazal region, indicating an early separation of this region into two separately regulated developmental domains (one for each of the two integuments). Second, the *ino* mutant phenotype highlights the fact that Arabidopsis ovules must also establish a lateral developmental pattern in a direction perpendicular to the ovule axis. This is first apparent at stage 2-III of ovule development when the outer integument primordia of wild-type ovules uniformly initiate on the abaxial sides of ovule primordia (ROBINSON-BEERS *et al.* 1992; SCHNEITZ *et al.* 1995). The earliest visible effect of *ino* is an apparent reversal of this polarity, resulting in initiation of the outer integument on the adaxial side of each ovule primordium. We note that the polarity of only the integument is reversed as bending at the base of the nucellus and curvature of the funiculus are both in the normal adaxial and abaxial directions, respectively (Figure 1). Examples of polarity mutations, such as the tissue polarity mutants of Drosophila (ADLER 1992), have been described in other systems, but we are not aware of other examples of such polarity shifts in higher plants.

The lack of further significant development of the misplaced outer integument primordia of *ino* mutants could indicate that the INO gene product is essential for subsequent steps in outer integument development. Alternatively, the failure to develop could be an indirect effect resulting from the misorientation of the integument primordium. We have previously shown that the Arabidopsis *SUP* gene is essential for normal asymmetric expansion of the outer integument (GAISER *et al.* 1995). The specific role of the *SUP* gene in this process is suppression of expansion of the outer integument on the adaxial side of the ovule. Thus, it seemed possible that by placing the larger part of the outer integument primordium on the adaxial side of the ovule it could fall under this inhibitory influence. The observation that *sup ino* double mutant plants have ovules indistinguishable from *ino* single mutants shows, however, that this is not the case (GAISER *et al.* 1995). Thus we do not yet have information to differentiate between the two different hypotheses for cessation of outer integument development in *ino* mutants.

ANT and *BEL1* appear to be responsible for integument initiation (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996; this work) and integument identity (REISER *et al.* 1995), respectively. Our demonstration that mutations in either of these genes are epistatic to *ino* shows that these two events occur before the action of *INO* on orientation of the outer integument primordium, and that all three events are part of a single pathway. In contrast, effects of *sin1* were essentially directly additive with those of *ino* indicating that these two genes act on relatively independent aspects of ovule development.

The effects of the *ats* and *ino* mutations were also largely additive but the phenotype of the double mutant aids in interpretation of both mutations. In the double mutant, it appears that fusion of the inner integument (mediated by the *ats* mutation) to the abortive outer integument (caused by the *ino* mutation) prevents normal inner integument development. One model to explain this is that the *ats* mutant lacks the outer layer of the inner integument. This layer may be necessary for supporting normal inner integument growth. In the *ats* mutant, the supportive function of the outer layer of the inner integument may be compensated by attachment to a normally growing outer integument. The absence of this compensating tissue in the double mutant would then prevent normal growth of either integument.

Both *ino* and *ats* mutants produce normal embryo sacs (Figure 2B) (LEON-KLOOSTERZIEL *et al.* 1994), indicating that each of these genes is dispensable for normal development of this structure. In contrast, the double mutant fails to produce an embryo sac (Figure 2E).

One possible explanation for this is that each of these genes plays a role in embryo sac development, but that either gene can compensate for the absence of the other in this process. An alternative, simpler explanation is that the absence of an embryo sac in the double mutant is an indirect effect of the other effects of the combination of these two mutations. In contrast to either single mutant, the nucellus of the double mutant remains uncovered by integumentary layers. Thus, it is possible that the presence of a sheathing integument is a necessary precondition to formation of a normal embryo sac. We note that all other currently described mutations that result in an exposed nucellus (*ant, bel1,* and *sin1*) are defective in embryo sac formation.

The altered surface topography of Ino$^-$ seeds indicates that the normal differentiation of the cells on the outside of a seed is not directed simply by the cells sensing their location on the outside of this structure. Rather, cells on the outside of a seed must have derived from the outer integument to correctly differentiate to form the outside of the seed coat. This indicates that lineage, as well as position is an essential component of seed coat development.

Effects and genetic interactions of *ant* mutations: In agreement with prior observations, the strongest *ant* mutations result in near complete absence of integument development (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996; this work). This indicates that the *ANT* gene acts very early in integument initiation. However, in most genetic backgrounds, even putative null mutants (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996) and our strongest mutant (*ant-4*), are able to form small integumentary ridges. It therefore appears that ANT does not control the very earliest steps in integument development. Rather, some other gene(s) must establish the anlage to the integument primordia (the region destined to give rise to these structures) and facilitate initiation of expansion. This is consistent with the observation that specificity of initial expression of the *BEL1* gene in the chalaza of the ovule is unaltered in *ant* mutants (REISER *et al.* 1995). An early role of the ANT gene product is then to promote the continuation of integument expansion. The arrest in integument development is not due to a general arrest of all aspects of ovule development as cellular differentiation continues in both the funiculus and nucellus of *ant* ovules (Figure 4).

While a megasporocyte differentiates in the nucellus of a severe *ant* mutant ovule, this cell commonly fails to undergo meiosis and never forms a normal embryo sac (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996) (Figure 2C). *In situ* hybridizations with the *ANT* cDNA showed that this gene is not expressed in the nucellus at the time of megasporocyte differentiation (ELLIOTT *et al.* 1996). Thus, as hypothesized for the *ino ats* double mutant, the failure of embryo sac formation in *ant* mutants may be a secondary effect of the absence of integuments (ELLIOTT *et al.* 1996; KLUCHER *et al.* 1996; this work).

Additional aspects of *ANT* gene function were revealed by the phenotypes of our weaker *ant* alleles. The primary differences between alleles are in the degree of suppression of integument expansion. Thus, integument growth may depend on the relative quantity of ANT activity that is present. A common feature of all of our alleles is a failure to form two separate integuments. The size of the region producing the integumentary ridge in weak *ant* alleles has led us to conclude that this single structure results from failure to separate the regions that would give rise to both integuments, rather than from the absence of one integument. Thus, the single integument of weak *ant* alleles is similar to that formed through fusion of the two integuments in *ats* mutants (LEON-KLOOSTERZIEL *et al.* 1994). It is also possible that the single integument-like structure that replaces the integuments in *bel1* mutants derives from the region normally giving rise to both integuments (RAY *et al.* 1994; GASSER 1996). Thus, emergence of two primordia from the anlage to the integuments appears to be a discrete step in integument formation that requires the function of at least two, and possibly three, different genetic loci.

With respect to ovule development, strong *ant* alleles were epistatic to the tested alleles of *ino, bel1, sin1,* or *sup.* While we have no verification that any of these alleles are true nulls, the apparent epistasis is in agreement with the morphological and anatomic analyses that indicate that the effects of strong *ant* mutations on integuments manifest themselves before the effects of any of the other tested mutations. The absence of integuments resulting from a strong *ant* mutation would clearly mask the effects of all of these other mutations that affect only the form of the integuments. Thus, *ANT* appears to be the earliest acting gene yet identified in Arabidopsis ovule development.

The *sin1* mutation accentuates the narrow petal phenotype of *ant-4* mutants, indicating that *ANT* and *SIN1* are partially redundant in terms of petal development. Because petals of *ant SIN1* plants show more extreme phenotypic effects than those of *ANT sin1* plants, *ANT* plays a more critical role in petal development than *SIN1.* The phenotype of *ant sin1* double mutants also shows that the function of ANT interacts to some extent with that of SIN1 in stamen development. The stamens of *sin1* mutants were shown to mature later and produce significantly less pollen (in the Landsberg *erecta* background, see below) than wild-type stamens (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994). *ant-4 sin1* double mutants produce even less pollen than *sin1* mutants and exhibit the frequent absence of lateral stamens characteristic of *ant* mutants. Prior work on *ant* mutants has shown that *ANT* and *AP2* have partially redundant activities in primordia formation and development of floral organs (ELLIOTT *et al.* 1996). Thus, *ANT, AP2,* and *SIN1* may all act together to promote proper growth of several different floral organs.

All of our studies of interactions of *ant* and *sin1* mutations were performed in the Landsberg *erecta* back-

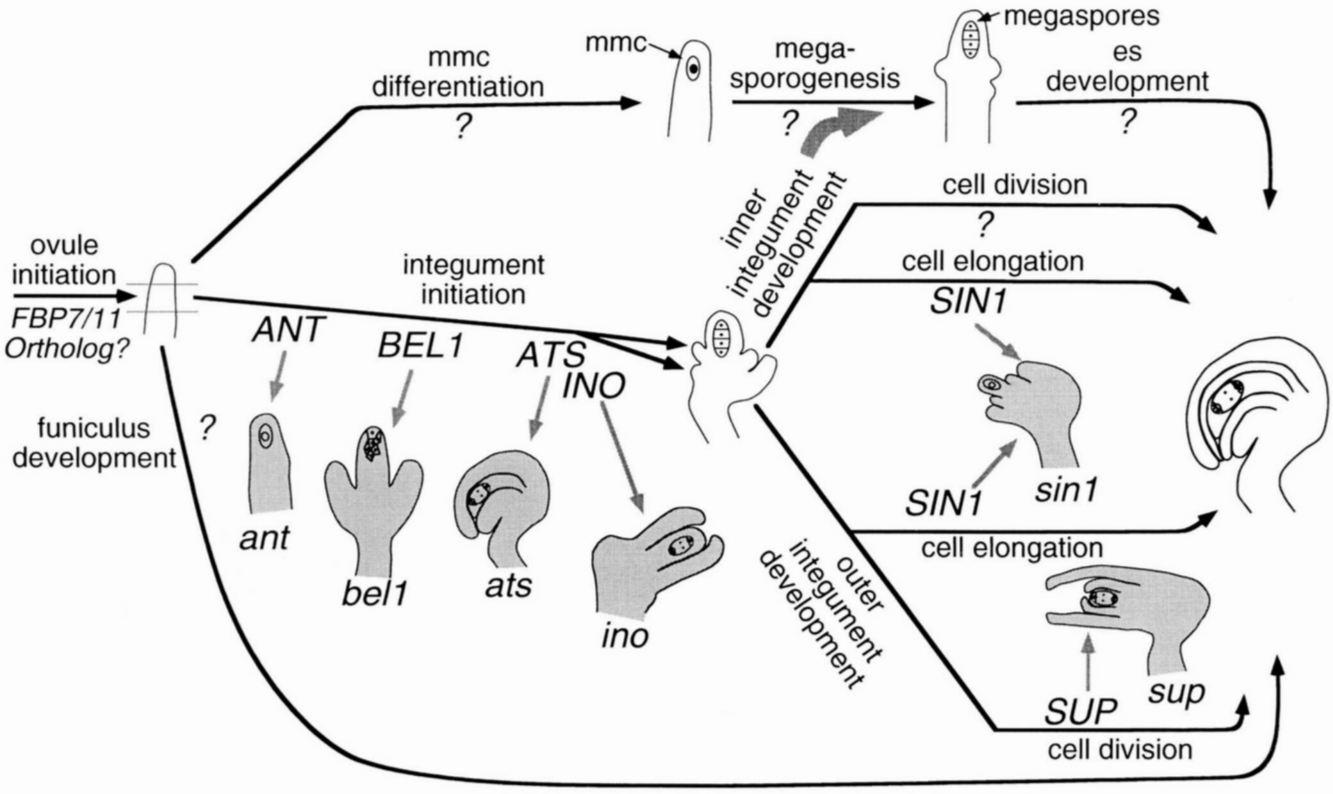

FIGURE 7.—Model for the genetic control of ovule development. The overall pathway of Arabidopsis ovule development is represented in this figure as a series of key developmental stages connected by the processes that are required to achieve each stage. The developmental progression occurs from left to right and is subdivided into the three main parallel pathways of nucellar, chalazal, and funicular development. The chalazal development pathway is further subdivided into separate pathways for each of the two integuments. Within each integument there is separate control of cell division and cell expansion. Black arrows indicate the normal progression through the ovule development pathway. The processes that the arrows represent are described in Roman letters next to the arrows. Labels in upper case italics indicate the points of action of wild-type genes in the pathway. Thin gray arrows, labeled in lower case italics, indicate deviations from the pathway caused by specific mutations. The phenotypes of the mutants at anthesis are illustrated in gray to show the results of the deviations from the wild-type pathway. The thick gray arrow represents the promotion of embryo sac development by the inner integument. Question marks indicate genes predicted to exist by the model which have not yet been identified. See text for further details of phenotypic effects of the mutations. es, embryo sac; mmc, megaspore mother cell.

ground. LANG *et al.* (1994; A. RAY, personal communication) have shown that *er* accentuates the effects of the *sin1* mutation. While we have not yet produced all combinations of these genes, *ant ER* plants show the same petal phenotype observed in *ant er* plants (data not shown). Thus the *ER* gene either plays no role, or plays a secondary role, in the interactions of *ant* and *sin1* on petal development.

A model for genetic regulation of ovule development: Figure 7 shows a preliminary model of the genetic control of ovule development. This model is based on our analysis of *ino* and *ant* mutants and double mutants and on prior results from other studies on ovule development mutants. Because differential effects of mutations on such structures as the inner and outer integuments indicate that the different parts of ovules develop in at least partial independence, the model branches to include a number of parallel pathways. The first branching in the pathway occurs at control of development of the nucellus (and megagametophyte), the chalaza, and the funiculus consistent with the three zones of the ovule primordium proposed in previous models (REISER *et al.* 1995; SCHNEITZ *et al.* 1995). The chalazal branch of the pathway further subdivides into branches representing the separate pathways for inner and outer integument development.

While the *ant, sin1,* and *bel1* mutations all affect embryo sac formation, the fact that *ANT* and *BEL1* are not expressed in the nucellus during the early formation of this structure argues against a direct role for these genes in this process (REISER *et al.* 1995; ELLIOTT *et al.* 1996). In addition, the *ino* and *ats* mutations affect this process only when present in combination (Figure 2E). The presence of a naked nucellus in all these mutant phenotypes is in agreement with the hypothesis that signals from a surrounding (inner) integument are necessary for normal embryo sac development (REISER *et al.* 1995; ELLIOTT *et al.* 1996; this work). The role of the integuments in this process must begin after differentiation of the megaspore mother cell as this process can occur in all of these mutants. The model therefore includes promotion of meiosis and embryo sac develop-

ment by the inner integument following megaspore mother cell formation. The contribution must be more than just the presence or absence of a surrounding layer, as embryo sac development proceeds further in the *bel1* mutant than in the *sin1* mutant (ROBINSON-BEERS *et al.* 1992) despite the fact that the inner integument is more fully developed in the *sin1* mutant.

All six Arabidopsis genes included in our model act in the integument development branch of the pathway. As noted above, *ANT* appears to act earlier in this process than the other currently described genes. *BEL1* acts before, and *ATS* acts in, the subsequent step of separation of the inner and outer integuments (ROBINSON-BEERS *et al.* 1992; LEON-KLOOSTERZIEL *et al.* 1994; RAY *et al.* 1994). *bel1* mutations subsequently lead to a complete deviation from the normal ovule development pathway (ROBINSON-BEERS *et al.* 1992; MODRUSAN *et al.* 1994; RAY *et al.* 1994; REISER *et al.* 1995). In contrast, the *ats* mutant produces viable seeds (LEON-KLOOSTERZIEL *et al.* 1994), indicating that its effects are specific to the integument separation process and that later steps in ovule development proceed normally despite the failure in separation. The *INO* gene must act after the *BEL1* gene, because of the epistasis of *bel1* with respect to *ino,* but appears to first act at approximately the same time as the *ATS* gene.

We have previously shown that the loss of integument identity in *bel1* mutants was associated with elevated expression of *AG* mRNA, and that induced ectopic *AG* expression could produce an apparent phenocopy of the *bel1* mutation. On the basis of these observations, we proposed that a primary function of BEL1 was suppression of *AG* during integument development (RAY *et al.* 1994). However, the more recent observation that there is substantial overlap between the regions of expression of *BEL1* and *AG* early in integument development is inconsistent with this simple model (REISER *et al.* 1995). Thus, it appears that the absence of BEL1 activity can directly lead to loss of integument identity and that ectopic expression of AG produces a similar loss of integument identity through a separate mechanism. Further experimentation may resolve the apparent discrepancy between these two sets of data, but for now we are unable to assign a specific role to *AG* in the ovule development pathway.

The other genes act in later stages of integument development. *SIN1* has been shown to be necessary for proper elongation of cells of both integuments but does not govern cell number in these structures (ROBINSON-BEERS *et al.* 1992; LANG *et al.* 1994). This indicates that the processes of cell division and elongation in integuments are at least partially under separate control, and, therefore, these processes are separated in our model.

SUP and *INO* are both required for proper cell division in the outer integument and have no direct role in inner integument development (GAISER *et al.* 1995; this work). Because *ino* is epistatic to *sup* with respect to ovule development (GAISER *et al.* 1995), *INO* appears to act earlier in outer integument development than does *SUP.* The differential effects of these two genes on the two integuments demonstrate that inner and outer integument development represent independent parallel events and they are therefore shown separated in our model.

Our model is consistent with recent molecular data. The expression of *ANT* in the placenta (ELLIOTT *et al.* 1996) precedes that of *BEL1* in ovule primordia (REISER *et al.* 1995; KLUCHER *et al.* 1996), consistent with our epistatic analysis and the placement of the two genes in the pathway.

Certain alleles of the Arabidopsis *apetala2* (*ap2*) mutation have been shown to have effects on early aspects of ovule development (MODRUSAN *et al.* 1994). However, *ap2* plants produce both aberrant and normal ovules. In addition, other apparently strong *ap2* alleles result in normal ovules and only affect later stages of development of these structures during seed formation (JOFUKU *et al.* 1994). Because of this variability in the effects of mutations in this gene, we did not assign a role to *AP2* in our model of regulation of ovule development.

Two genes, *FBP7* and *FBP11,* which appear to be involved in ovule initiation and identity in petunia, have recently been characterized (ANGENENT *et al.* 1995; COLOMBO *et al.* 1995). These two genes encode MADS box transcription factors (ANGENENT *et al.* 1995), and Arabidopsis is known to contain a family of related genes (MA *et al.* 1991; ROUNSLEY *et al.* 1995; SAVIDGE *et al.* 1995), including one (*AGL11*) that is very similar in sequence and expression pattern to *FBP7* and *FBP11* [(ROUNSLEY *et al.* 1995; ANGENENT and COLUMBO 1996), YANOFSKY Lab WWW Pages (URL at http://glamdring.ucsd.edu/ others/yanofsky/home.html)]. We, therefore, hypothesize that *AGL11,* or another ortholog of *FBP7/11,* is involved in activation of the Arabidopsis ovule development program.

To accommodate new data, our proposed model is more complex than those previously published (REISER *et al.* 1995; SCHNEITZ *et al.* 1995; ANGENENT and COLOMBO 1996). The most significant changes in the model are the increase in the number of developmental zones within the ovule primordium and the branched topology indicating parallel independent developmental pathways. Our model must be considered preliminary as the epistatic analysis between the currently identified genes is not yet complete. However, the model is testable as it predicts the existence of additional regulatory genes and the patterns of expression and timing of activity of known genes. Further screening for additional mutants, in combination with genetic and molecular characterizations, will allow testing these predictions to further refine understanding of genetic control of ovule development.

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